Exhibit A

#31
5-1-03

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Davis et al.

Serial No: 09/339,818

Filed: June 25, 1999

For: Linear Cyclodextrin Copolymers

Attorney Docket No. CTCH-P02-012

Art Unit: 1623

Examiner: L.E. Crane

Commissioner of Patents
Washington, D.C. 20231

Declaration Under 35 U.S.C. §1.132 of Ronald Breslow

Sir:

I, Ronald Breslow, of 275 Broad Avenue, Englewood, New Jersey, hereby declare as follows:

1. I am a Professor of Chemistry at Columbia University in New York, New York. I received a B.A. in Chemistry from Harvard University in 1952, an M.A. in Chemistry from Harvard University in 1954, and a Ph.D. in Chemistry from Harvard University in 1955, where I conducted my graduate research in the laboratory of the Nobel Laureate, Professor Robert Woodward. Subsequently, I was a post-doctoral fellow in the laboratory of the Nobel Laureate Lord Todd in the department of Organic Chemistry at Cambridge University. In 1956, I came to Columbia University as an Instructor in Chemistry. I am now one of twelve University Professors, and a former Chairman of the Chemistry Department.

2. I am a member of the U.S. National Academy of Sciences (Chairman of the Chemistry Division 1974-77), of the American Academy of Arts and Sciences, and of the American Philosophical Society (member of the Council, 1987-92), as well as other scientific societies including the New York Academy of Sciences. I am a Foreign Fellow of the Indian National Science Academy, an Honorary Member of the Korean Chemical Society, an Honorary Member of the Royal Society of Chemistry of Great Britain, a Foreign Member of the Royal Society of Britain, a Fellow of the World Innovation Foundation, and an Honorary Member of the Chemical Society of Japan. I have been the Chairman of the Board of Scientific Advisors of the Alfred P. Sloan Foundation, and a member of the Board

of Trustees of Rockefeller University. I am on the editorial board of a number of scientific journals, and have held over 150 named and visiting professorships.

3. My major scientific awards include the American Chemical Society Award in Pure Chemistry (1966), the Fresenius Award of Phi Lambda Upsilon (1966), the Baekeland Medal (1969), the Centenary Medal (1972), the Harrison Howe Award (1974), the Remsen Prize (1977), the Roussel Prize in Steroids (1978), the James Flack Norris Prize in Physical Organic Chemistry of the American Chemical Society (1980), the Richards Medal (1984), the Arthur C. Cope Award (1987), the Kenner Award (1988), the Nichols Medal (1989), the National Academy of Sciences Award in Chemistry (1989), the Allan Day Award (1990), the Paracelsus Award and Medal of the Swiss Chemical Society (1990), and the U.S. National Medal of Science (1991). I was recently named one of the top 75 contributors to the chemical enterprise in the past 75 years by Chemical & Engineering News (1997), and won the Priestley Medal (1999). In 2000, I won the New York City Mayor's Award in Science and in 2002 I received the ACS Bader Award in Bioorganic or Bioinorganic Chemistry and the Esselen Award for Chemistry in the Public Interest. I have also received the Mark Van Doren Medal of Columbia University and the Columbia University Great Teacher Award. I was president of the American Chemical Society in 1996.

4. For more than 30 years now, I have had considerable involvement in research directed to the chemistry of cyclodextrins. In particular, my research has included the use of a variety of different cyclodextrin derivatives in various synthetic schemes, and I have used a wide range of different cross-coupling and other linking reactions to build macromolecules including cyclodextrin moieties. I have published more than 45 scientific papers, most in peer-reviewed journals, pertaining to my research in the field of cyclodextrins. In view of my experience with this research, I am one of the leading investigators in the field of cyclodextrin chemistry.

5. I have reviewed the specification of Kosak U.S. Patent Application Serial Number 09/067,921 (Attached as Exhibit 1, herein the "Kosak Application"). I understand that the Kosak Application was filed April 29, 1998. As my biography above indicates, I am aware of what the state of the art of the relevant cyclodextrin chemistry was at the time the Kosak Application was filed.

6. I have reviewed the specification of the Davis et al. U.S. Patent Application Serial Number 09/339,818 (attached as Exhibit 2, herein the "Davis Application"). I understand that the Davis Application was filed June 15, 1999, and claims priority to US Patent 6,048,736 (filed December 2, 1998) and U.S. Provisional Application Serial Number 60/091,550 filed July 1, 1998 (attached as Exhibit 3, herein the "Davis Provisional Application"). I am aware of what the state of the art of relevant cyclodextrin chemistry was at the time the Davis Application and its priority applications were filed.

7. I have also reviewed the outstanding Office Action dated October 22, 2002, (Attached as Exhibit 4, herein the "Office Action") that was issued in connection with the Davis Application, and the pending claims of the Davis Application (Attached as Exhibit 5). I understand that the Examiner has rejected certain of the pending claims on the grounds that the Kosak Application teaches or suggests chemistry that would produce a water-soluble, linear cyclodextrin polymer as claimed in the Davis Application. I also understand that the Examiner has rejected certain of the pending claims of the Davis Application on the grounds that the claims cover a broad range of comonomers (linkers), and argued that the Davis Application does not provide sufficient guidance in how to choose, make, or test a linker within the scope of those rejected claims. I respectfully disagree with the Examiner's grounds for these rejections.

The Kosak Application

8. I have reviewed the Kosak Application with regard to polymerization reactions involving cyclodextrin. From my review, there are only a few instances in which the Kosak Application discusses cyclodextrin polymers.

A. Pages 12, 77, 88, and 102, for example, refer only to cyclodextrin polymers generally, and none of those pages suggest that Kosak was referring to linear cyclodextrin copolymers or polymers of any particular structure whatsoever.

B. Page 31 of the Kosak Application provides a synthesis protocol for a cyclodextrin polymer:

"The basic procedure was to combine 2 ml of 4.4% cyclodextrin in water, 0.1 ml of 2 N NaOH and .116 ml of 1,4 butanediol diglycidyl ether

(BDE) while mixing and incubating at 50 °C. The molar ratio of BDE to cyclodextrin was about 5:1.

"After about 4 hours, a 0.5 ml aliquot of the mixture was mixed with 0.2 ml of lysine (0.8 M in water, neutralized) for about 1.5 hours. The CD polymer was then fractionated on a column of Sephadex® G-25 (21 x .8 cm) equilibrated with distilled H_2O and pre-calibrated with free cyclodextrin."

Under those conditions, it is highly unlikely any significant amount of disubstituted cyclodextrins would form, and such disubstituted cyclodextrins would be necessary to form linear polymers when treated with lysine. In particular, it is evident from the stoichiometry of the reaction, i.e., 5 equivalents of BDE relative to cyclodextrin, that the above procedure was plainly intended to produce random, highly crosslinked polymer networks, not linear polymers. There is no more than a remote possibility that linear polymers would be generated under these conditions, and if then, only as an insubstantial contaminant of the reaction.

9. Reading the Kosak Application in its entirety, and taking into account the particular discussions concerning polymerization reactions involving cyclodextrins, I conclude that the Kosak Application contemplated only random highly crosslinked networks. I find no support in the Kosak Application to suggest that the inventor contemplated structures or reaction schemes that were intended or can be expected to produce linear polymers. Neither do I believe that an ordinary chemist would be motivated to produce linear polymers based on the teachings of the Kosak application.

The Davis Application

10. One aspect of the Davis Application relates to a method for synthesizing water-soluble, linear cyclodextrin copolymers using functionalized cyclodextrins and comonomers as starting materials. As of the priority date of the Davis Application, namely July 1, 1998, the synthesis described in that application represented an elegant synthetic approach to generating linear cyclodextrin-containing polymers. One reason I consider the synthetic schemes of the Davis

Provisional Application to be an elegant approach is that, based on the teachings of the application, the average organic chemist would have readily recognized in July 1998 that a wide range of functionalized cyclodextrin and comonomers would be readily adaptable for use in the polymerization schemes set out in the Davis Provisional Application. The reaction chemistry underlying the synthesis of linear cyclodextrin copolymers, once revealed by the teachings of the Davis Provisional Application, was already well characterized in the literature. It is my opinion that in July 1998 the average organic chemist would have been able to determine a priori whether a particular set of functionalized cyclodextrins and comonomers would produce a linear cyclodextrin copolymer, and have been able to test combinations without any undue experimentation. The basis for this conclusion follows.

11. The Davis Provisional Application describes in detail a diverse set of comonomers that can be used to join functionalized cyclodextrins to form linear cyclodextrin copolymers. The paragraph bridging pages 11 and 12, for example, makes clear that the comonomer A precursor has two functional groups that will react to form a bond with a cyclodextrin monomer. Page 12 provides a list of suitable functional groups and enumerates a number of suitable comonomers. Pages 16-19 of this application point out that the polymerization can be carried out in either of two polarities, i.e., selecting a comonomer precursor including two nucleophilic groups to react with a functionalized cyclodextrin including two leaving groups, or selecting a comonomer precursor including two leaving groups to react with a functionalized cyclodextrin including two nucleophilic groups. Reactions of this sort were well known and routinely, reliably, and predictably used to link molecules together by July 1, 1998. Thus, at that time, an ordinary chemist would readily have been able to select comonomer precursors that would satisfy the criteria provided in the Davis Provisional Application and that would react with a given functionalized cyclodextrin monomer to form a linear copolymer. No more than routine experimentation would be required for an ordinary chemist at that time to select suitable comonomers and cyclodextrin monomers, or to react them together to form linear copolymers according to the teachings of Davis Provisional Application.

12. By July 1, 1998, a substantially large number of difunctionalized linkers had been described in the literature, and the versatility of this class of linkers had been well established. For instance, several of my own papers published before July 1, 1998,

attached as Exhibit 6, demonstrate the use of a wide variety of functional groups and reactions for linking moieties to cyclodextrin rings. While these references don't suggest the generation of linear cyclodextrin copolymers, they do establish the state of the art of relevant linker chemistry. Based on the teachings of the Davis Provisional Application, and in view of the substantial body of literature on linker chemistry, an organic chemist of ordinary skill would have been able to select comonomer precursors suitable to use with a given functionalized cyclodextrin to form water-soluble linear cyclodextrin copolymers of widely varying structures, as taught by the Davis Provisional Application. No undue experimentation would be necessary to adapt these teachings or other related teachings in the art to the preparation of linear cyclodextrin copolymers as described in the Davis Provisional Application.

13. Thus, I believe that by July 1, 1998, it would have been no more than routine experimentation for an ordinary organic chemist to design and prepare a wide range of linear cyclodextrin copolymers as claimed in the Davis Application relying on the teachings of the Davis Provisional Application and the general knowledge available in the art at that time.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code and that willful false statements may jeopardize the validity of this Application for Patent or any patent issuing thereon.

Ron Breslow

Dated: 4/2/03

Signature: 

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Davis et al.	
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Commissioner of Patents
Washington, D.C. 20231

Declaration Under 35 U.S.C. §1.132 of Mark E. Davis

Sir:

I, Mark E. Davis of Pasadena, California, hereby declare as follows:

1. I am a co-inventor of the abovementioned application which teaches and claims water-soluble linear cyclodextrin copolymers.

2. Since the filing of the abovementioned application, my research group has made water-soluble linear cyclodextrin copolymers using a wide variety of comonomers following the teachings of the application. In every instance, we have been able to predict, based on reactant structures and general principles of chemical reactions, what combinations of functionalized cyclodextrins and comonomers will produce a water-soluble linear cyclodextrin copolymer. Indeed, every comonomer we have selected has resulted in a water-soluble linear cyclodextrin copolymer that exhibits the useful properties taught in the specification of the abovementioned patent application.

3. The level of skill required to make and test combinations of functionalized cyclodextrins and comonomers is relatively low, in that it can be done by a chemist with substantially less than doctoral training. In my research group, the preparation of water-soluble linear cyclodextrin copolymers is routinely carried out by graduate students with little more experience and training than a bachelor's degree, regardless of the choice of comonomer.

4. The papers of Exhibit D, which have been made public or are in press, describe experiments performed under my direction. These papers describe various water-soluble

linear cyclodextrin copolymers made following the teachings of the abovementioned application. These examples substantiate the allegations of the application that a wide range of comonomers can be used, illustrating the flexibility in steric and electronic characters that the copolymerization chemistry can tolerate.

5. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code and that willful false statements may jeopardize the validity of this Application for Patent or any patent issuing thereon.

Mark E. Davis

Dated: April 11, 2003

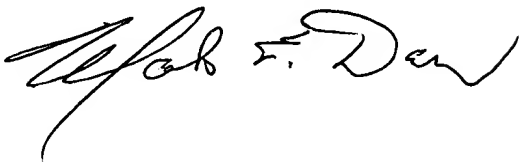
Signature: 

Exhibit D

Antitumor Activity of Systemic Delivered Camptothecin Conjugates of Linear, Cyclodextrin-Based Polymers

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ABSTRACT SUMMARY

New linear, cyclodextrin-based polymers (CDP) are synthesized with various average molecular weights. Camptothecin (CPT) is conjugated to these polymers via different linking chemistries and with variable loadings, and used to systemically deliver CPT to tumors in nude mice. The effects of polymer molecular weight (MW) and linkage on toxicity and tumor growth inhibition are described. All the CDP-CPT conjugates show significant antitumor activities. Conjugates with MW higher display stronger tumor inhibition activity than those of low MWs. The CDP-CPT conjugate with a glycine linker is less toxic than that with a triglycine linker.

INTRODUCTION

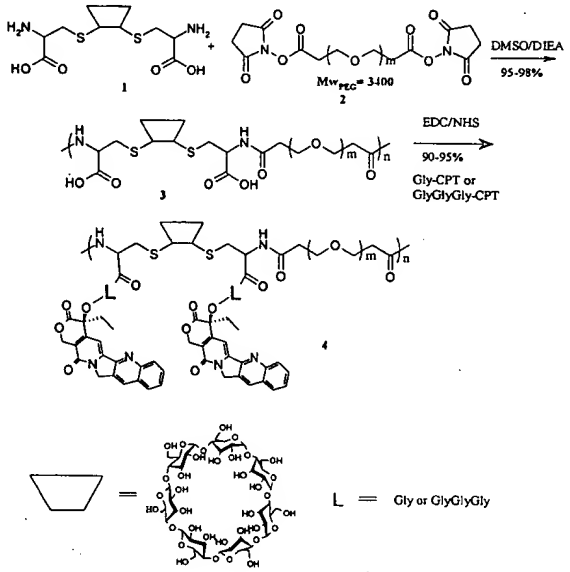
Numerous small molecules have been shown to be effective antitumor agents. However, many of these molecules are limited in clinical effectiveness due to their high toxicity, low solubility, or other poor pharmaceutical profiles. It has been demonstrated that conjugation of an anticancer drug to a water soluble polymer can greatly enhance its water solubility and reduce its cytotoxicity. In addition, high molecular-weight (MW) polymers can substantially enhance a drug's half-life and stability during circulation, and increase the accumulation of drug in tumor tissue through enhanced permeability and retention (EPR) effects. Therefore, development of polymeric drug delivery vehicles has attracted much attention.[1-8]

Camptothecin (CPT), an anticancer agent that can selectively inhibit the topoisomerase I enzyme, is water insoluble and highly toxic.[9-12] CPT has a pH-dependent equilibrium between its lactone form (below pH 4) and carboxylate form (above pH 7). The lactone is essential for anti-cancer activity while the carboxylate form is favored at physiological pH. Human serum albumin (HSA) preferentially binds the carboxylate form of CPT and forces the distribution of CPT to further favor this form. This results in rapid lactone-ring opening and a loss of antitumor function.[9]

Cyclodextrin (CD) is well known for its low toxicity and non-immunogenicity. CD-based polymers have recently been synthesized and used as non-viral vectors for systemic gene delivery.[13-15] Herein, we report the synthesis and properties of linear, CD-based polymers (CDPs) for systemic delivery of CPT.

EXPERIMENTAL METHODS

I. Synthesis of CDP-CPT conjugates



CDP (**3**) was synthesized by polymerizing a β-CD-dicysteine (**1**) with PEG-DiSPA (**2**). The molecular weight of the CDPs (**3**) can be controlled by adjusting polymerization time. Polydispersities of these polymers are usually between 1.5 and 1.8. The resulting polymer contains pendent carboxylate function groups for the coupling of CPT derivatives. CPT derivatives with two different linkers (glycine or triglycine) were conjugated to the pendent carboxylate groups on **3** using the conventional EDC/NHS method. **3** with two different molecular weights were chosen for CPT conjugation *via* either the glycine or the triglycine linker. Four different CDP-drug conjugates (**4A-D**) with variable polymer MW, linking chemistries, or weight percent of drug loading were synthesized and are listed in the following table.

Conj	Mn of **3** (x 10^{-3})	Mw/Mn	Linker	CPT (wt%)
4A	58	1.67	trigly	6
4B	26	1.62	trigly	10
4C	58	1.67	gly	6
4D	58	1.67	trigly	10

For measurement of *in vitro* toxicity, the MTT assay was conducted with PC3 cell, at concentrations of polymer-drug conjugate from 200 ng CPT/mL to 20 μg CPT/mL.

II. Determination of maximum tolerable dose (MTD)

The measurement of MTD was conducted using female Charles River nude mice. The MTD was determined for all drug conjugates except for **4A**, which is identical to **4D** except that it has lower drug loading. The D5W solution of **4B-D** was freshly prepared before each injection. The dose for the treated groups covers a broad range from 2.25 mg CPT/kg to 54 mg CPT/kg. Dosing was administrated intravenously by tail vein injection at day 1, 5, and 9. Three mice were used in each treatment group. The dosing volume was determined based upon a ratio of 200 μL for a 20 g mouse, and was scaled appropriately according to actual body weight (BW) of the mice. The BW of mice was followed daily for the first 5 days and then twice a week thereafter. The MTD was defined as the highest administrated dose that resulted in a decrease of mean group BW of greater than 20% or the highest administrated dose that resulted in the death of any animal in that group.

III. Antitumor efficacy study

The primary test for antitumor activity was also conducted in the same mice as used for MTD study bearing a single subcutaneous human colon tumor (LS174T). Tumor cells were implanted into the flank of mice approximately 14-18 days before dosing. The tumor volume for each mouse was determined by measuring the two dimensions of the tumor with calipers and calculated using the formula: tumor volume = (length x width^2)/2. Tumor volume was then converted to tumor weight assuming 1 mm^3 tumor in size is equal to 1 mg tumor in weight. Treatment began when the mean tumor size reached approximately 60-100 mg (day 1). D5W solution was injected into the mice for the control group. Also as control, CPT (9 mg/kg) and CPT-11 (100 mg/kg) was administrated i.p to the mice for the purpose of comparison with the drug conjugates **4A-D**. The solution of **4A-D** was freshly prepared each time before dosing in the same manner as described in the MTD study. Dosing was administrated intravenously by tail vein injection. All the polymer conjugates were tested at 9 and 4.5 mg CPT/kg except for **4B**, which was evaluated at 36, 18, and 9 mg CPT/kg. Determination of the dosing was based upon the MTD results. Each group was dosed at day 1, 5, and 9. Seven mice were treated in each group. The dosing volume was calculated in the same manner as did for the MTD measurement. The BW and tumor size of mice were measured daily for the first five days and then twice a week thereafter. The mice were euthanized when either the tumor weight was over 1.5 g or 60 days passed since the first dose. Both the MTD measurement and the antitumor efficacy study were conducted by Piedmont Research Center (Morrisville, NC).

RESULTS AND DISCUSSION

All of drug conjugates are very water soluble and show low cellular toxicity based on the *in vitro* MTT assay. The solubility of free CPT in water is only 4 μg/mL, however, **4A-D** can easily be dissolved in water at a concentration of 100 mg/mL. As a result, the solubility limit of CPT in aqueous solution is increased by more than three orders of magnitude when it is conjugated with CDP *via* either linker.

The MTD of **4B**, **4C**, and **4D** was determined to be 36 mg CPT/kg, 9 mg CPT/kg, and 9 mg CPT/kg, respectively. Based on the structural similarities between **4A** and **4D**, we expected that 9 mg CPT/kg should also be a tolerable dose for **4A**. These MTD data clearly show that polymer molecular weight has a large effect on the maximum tolerable dose.

The data of tumor weight are shown in Figure 1 for a dose of **4A-D** at 9 mg CPT/kg and compared to CPT and CPT-11 at 9 mg/kg and 100 mg/kg, respectively. All the conjugates give significant tumor growth inhibition as compared to the control (D5W) and CPT, and are at least as effective as CPT-11 when administrated at a dose approximately one order of magnitude higher. Group **4B** at the highest dose (36 mg CPT/kg) revealed similar antitumor activity as that observed at 9 mg CPT/kg as shown in Figure 1.

4D shows slightly stronger tumor growth inhibition than that of **4B** when both are administrated at 9 mg CPT/kg (Figure 1), indicating that polymer MW has an effect on the antitumor activity.

A comparison of **4A** and **4C** when both are administrated the same dose (9 mg CPT/kg) shows that linker can also affect the antitumor activity of conjugates (Figure 1). The drug conjugate with a glycine linker (**4C**) displays a slightly greater tumor inhibition response than that with a triglycine linker (**4A**).

Figure 1. *In vivo* Antitumor Activities of Polymer-Drug Conjugates at 9 mg CPT/kg

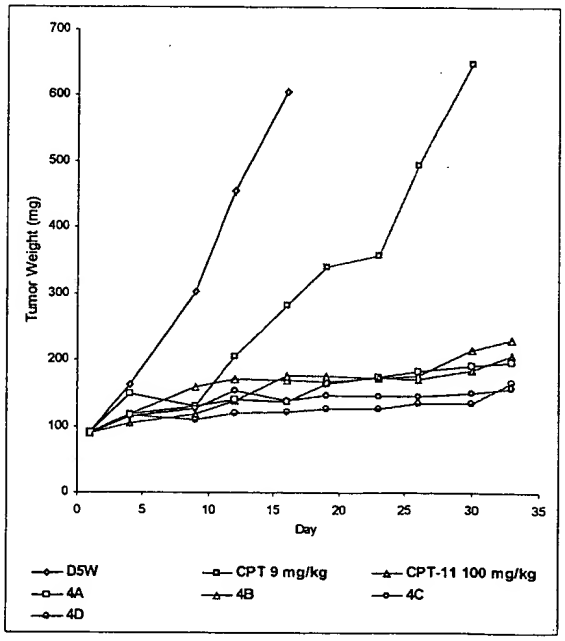
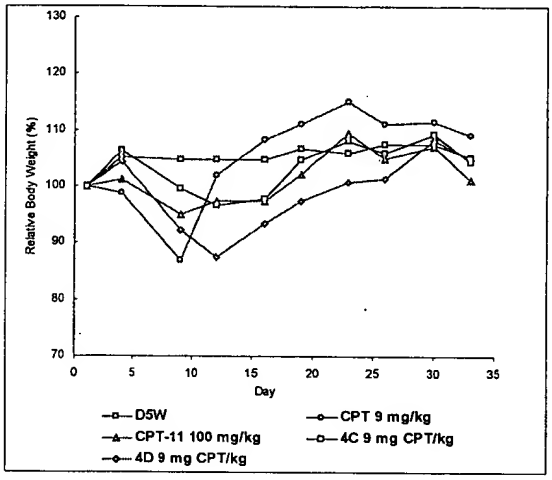


Figure 2 shows the relative BW change of the mice for each treatment group expressed as a percent of baseline. The mean BW loss was used as indication of toxicity. Two out of seven mice died in the free CPT group following the second dose. Although **4C** and **4D** show similar tumor inhibition responses at 9 mg CPT/kg (Figure 1), they display distinctly different toxicities when dosed at their MTD. One out of seven mice died in the **4D** group, while none of the animals died in the **4C** group. Maximum mean BW loss is much less for **4C** group than with **4D**. **4A** and **4B,** groups that contain GlyGlyGly peptide linker, also show similar BW loss profiles as that of **4D**. Toxicity of the conjugate containing the glycine linker **(4C)**, as measured by mean BW loss, is comparable to that of CPT-11.

Figure 2. Toxicity Analysis



CONCLUSIONS

A linear, cyclodextrin-based polymeric drug delivery system was prepared. CDP polymers were synthesized at various average molecular weights. Camptothecin (CPT) was conjugated to these polymers *via* either a glycine or a triglycine linker. The effects of polymer molecular weight (MW) and linkage chemistries on toxicity and tumor growth inhibition were investigated. All CDP-CPT conjugates tested generally display significant antitumor activities, showing the same or slightly better antitumor activity than CPT-11 at a dose approximately one order of magnitude lower. The conjugate of high MW polymer (Mn 58k) displays slightly stronger tumor growth inhibition than that of the low MW polymer (Mn 26k). The CDP-CPT conjugate with a glycine linker is found to be less toxic than that with a triglycine linker. These initial antitumor efficacy and toxicity studies demonstrate that CDP can be used for anticancer drug delivery to improve solubility, enhance antitumor activity, and reduce toxicity compared to parent compound. The tumor sizes and body weights of mice in all groups will be followed till the complete of this antitumor efficacy study. Biodistribution and pharmacokinetic analyses of these polymer-drug conjugates are underway.

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Structural Effects of Carbohydrate-Containing Polycations on Gene Delivery. 3. Cyclodextrin Type and Functionalization

Stephen R. Popielarski, Swaroop Mishra, and Mark E. Davis*

Chemical Engineering, California Institute of Technology, Pasadena, California 91125. Received January 21, 2003; Revised Manuscript Received February 13, 2003

Linear cationic β-cyclodextrin (β-CD)-based polymers can form polyplexes with plasmid DNA and transfect cultured cells. The effectiveness of the gene delivery and the cellular toxicity has been related to structural features in these polycations. Previous β-CD polycations were prepared from the cocondensation of $6^A,6^D$-dideoxy-$6^A,6^D$-diamino-β-CD monomers with other difunctionalized monomers such as dimethyl suberimidate (DMS). Here, the type of CD and its functionalization are varied by synthesizing numerous $3^A,3^B$-dideoxy-$3^A,3^B$-diamino-β- and γ-CD monomers. Both alkyl- and alkoxy-diamines are prepared in order to vary the nature of the spacing between the CD and the primary amines in the monomers. These diamino-CD-monomers are polymerized with DMS to yield amidine-based polycations. The nature of the spacer between the CD-ring and the primary amines of each monomer is found to influence both molecular weight and polydispersity of the polycations. When these polycations are used to form polyplexes with plasmid DNA, longer alkyl regions between the CD and the charge centers in the polycation backbone increase transfection efficiency and toxicity in BHK-21 cells, while increasing hydrophilicity of the spacer (alkoxy versus alkyl) provides for lower toxicity. Further, γ-CD-based polycations are shown to be less toxic than otherwise identical β-CD-based polycations.

INTRODUCTION

Numerous nonviral gene delivery studies are involved in elucidating the relationships between vector structure and transfection efficiency by modifying promising systems and assaying their performance. This ongoing research has demonstrated the significant influence of polycation structure on efficiency of gene delivery. Poly(ethyleneimine)s (PEIs) are a widely studied class of polycations for gene delivery. PEI molecular weight has been shown to affect both cytotoxicity and transfection efficiency (*1*, *2*). The charge density (*1*) and degree of branching (*3*) in the PEI backbone also significantly alter transfection efficiency in vitro. Furthermore, substituents grafted onto PEI affect the interaction of PEI with DNA as well as PEI/DNA polyplex interactions with cells (*4*–*6*, and references therein). Ionenes are another class of gene delivery vehicles whose structure has been related to stability of interaction with DNA (*7*) and to transfection efficiency (*8*). Structure–function studies have also been undertaken with systems based on chitosan (*9*), poly(L-lysine) (*10*), linear poly(amidoamine)s (*11*), polysaccharide–oligoamine conjugates (*12*), and others. It is clear from these reports that minor changes in structure of the gene delivery vehicle can have dramatic effects on the gene delivery efficiency and toxicity of the vector.

We have prepared families of linear, β-cyclodextrin-containing polycations (βCDPs) and have shown that these polymers can be used as gene delivery vectors (*13*, *14*). Cyclodextrins (CDs) are cup-shaped molecules formed of cyclic oligomers of glucose. Cyclodextrins comprised of 6, 7, and 8 glucopyranose units are called α-, β-, and γ-CD, respectively (β-CD is represented in Figure 1). There are three distinct hydroxyls per glucopyranose unit; two secondary carbons and one primary carbon bear these hydroxyls and they are labeled C(2), C(3), and C(6), respectively (Figure 1). The glucopyranose units are denoted alphabetically starting with 'A' and proceeding around the cyclodextrin ring (Figure 1).

Initial structure–function studies with βCDPs demonstrated the importance of intercharge spacing to transfection efficiency and toxicity (*14*). Significant effects on transfection efficiency were observed when the interamidine distance was reduced by just 2 Å. Upon the basis of this finding, we initiated a more complete structure–function investigation using linear, cyclodextrin-containing polycations. In part 1 of our study, we showed that cellular toxicity was related to the distance of the charge center from the carbohydrate unit (whether it be a cyclodextrin or trehalose), and that increasing polycation hydrophilicity provides decreasing toxicity (*15*). Part 2 of our work revealed that the type of charge center can dramatically change the delivery efficiency (*16*). With the βCDPs, amidine charge centers give greater gene delivery than quaternary ammonium charge centers. Here, we vary the type of cyclodextrin (β and γ) and the functionalization at the cyclodextrin, i.e., $3^A,3^B$-dideoxy-$3^A,3^B$-diamino-β- and γ-CD, as compared to the previously used $6^A,6^D$-dideoxy-$6^A,6^D$-diamino-β-CD, to prepare a distinct series of linear, cyclodextrin-containing polycations. Additionally, we report the effects of spacer length between the cyclodextrin and the charge center in order to make direct comparisons between otherwise identical β- and γ-CD-based polyamidines. The polycations were characterized and assayed for plasmid DNA (pDNA) binding, polyplex size and ζ-potential, and in vitro transfection efficiency and toxicity.

MATERIALS AND METHODS

β- and γ-Cyclodextrins were purchased from Wacker Biochem Corp. (Adrian, MI) and dried in vacuo at

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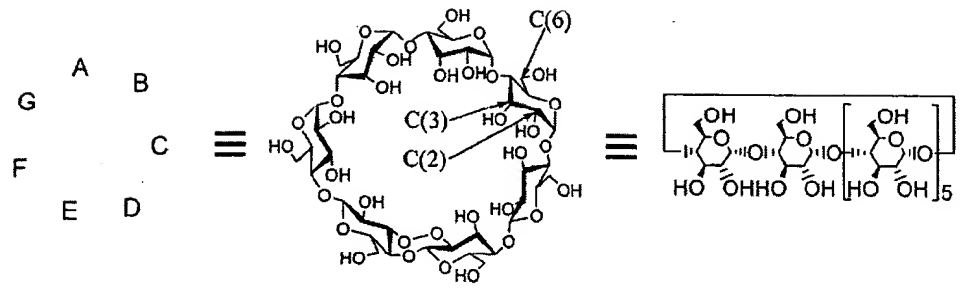
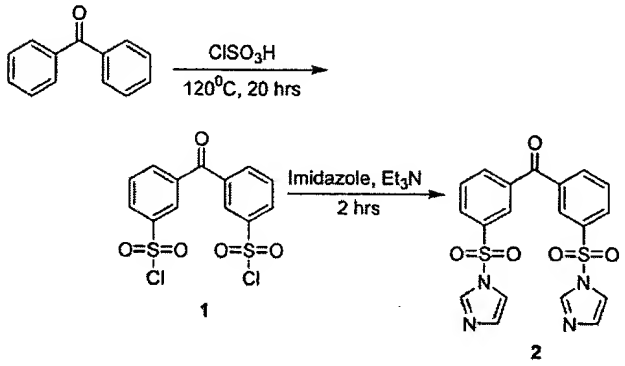


Figure 1. Representations and labeling of β-cyclodextrin.

Scheme 1



120 °C overnight before use. Chlorosulfonic acid (Alfa Aesar; Ward Hill, MA) was distilled before use. Dimethyl suberimidate·2HCl (DMS) was purchased from Pierce Endogen (Rockford, IL) and used without further purification. All other reagents were obtained from commercial suppliers and were used as received. Ion-exchange chromatography was run on a Toyopearl SP-650M (TosoHaas; Montgomeryville, PA) column (NH_4^+ form), and products were eluted with aqueous ammonium bicarbonate up to 0.4M. Thin-layer chromatography was performed on Silica Gel 60 F 254 plates (EM Separations Technology; Gibbstown, NJ) and the amino compounds were eluted with 5:3:3:1 *n*-PrOH:AcOEt:H_2O:NH_3(aq) and visualized by reaction with ninhydrin. Matrix-assisted, laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF-MS) was performed on a PerSeptive Biosystems Voyager DE PRO BioSpectrometry Workstation in the positive ion mode using a 2,5-dihydroxy benzoic acid matrix. NMR spectra were recorded on a Bruker AMX500 spectrometer as dilute solutions of either D_2O or DMSO-d_6. Dialysis was carried out using a 3500 molecular weight cutoff regenerated cellulose dialysis cassette (Pierce Endogen).

Synthesis of Benzophenone-3,3′-disulfonyl Chloride (**1**, Scheme 1). Benzophenone (26.06 g, 0.143 mol) was added in small portions to 190 mL (2.86 mol) of freshly distilled chlorosulfonic acid under an argon atmosphere. The solution was then heated to 120 °C with reflux. After 20 h at 120 °C, the cooled solution was added slowly to about 1000 g of ice in a 2 L Erlenmeyer flask. The slurry was poured into a separatory funnel then extracted with chloroform (350 mL then 300 mL) and washed with saturated $NaHCO_3$ (200 mL), water (200 mL), and saturated NaCl (200 mL, twice). The chloroform was removed under reduced pressure. The yellow solid obtained was recrystallized twice from chloroform/hexanes. First-crop yielded 30 g of off-white crystals; second-crop yielded 5.4 g. (65% yield). Anal. ($C_{13}H_8Cl_2O_5S_2$) C, H, Cl, S.

Synthesis of Benzophenone-3,3′-disulfonyl Imidazole (**2**, Scheme 1). **1** (13 g, 34.3 mmol) was dissolved in 150 mL of chloroform. Imidazole (4.95 g, 72.7 mmol) and Et_3N (10.2 mL, 73.2 mmol) were added. After about 30 min of stirring, 50 mL of dichloromethane was added to the slurry and allowed to stir for an additional 30 min. About 200 mL of dichloromethane was required to homogenize the reaction slurry, which was then washed with water (200 mL, twice) and dried with sodium sulfate. Benzophenone-3,3′-disulfonyl imidazole, **2**, was recrystallized from dichloromethane/ethyl acetate giving 13.9 g of colorless needles (92% yield). Anal. ($C_{19}H_{14}N_4O_5S_2$) C, H, N, S. NMR data were in agreement with published chemical shifts (*17*).

Synthesis of Cyclodextrin Polycations (**6a–d** and **7a–d**, Scheme 2). Syntheses of $2^A,2^B$-disulfonated β-cyclodextrin (*17*) (**3a**) and $2^A,2^B$-disulfonated γ-cyclodextrin (*18*) (**3b**) were carried out according to literature methods. NMR and mass spectra data were in agreement with published values (*17*, *18*).

Syntheses of $3^A,3^B$-di(aminoalkylamino)-β- and $3^A,3^B$-bis(aminoalkoxyamino)-γ-cyclodextrins (**4a–d** and **5a–d**) were carried out as exemplified by the following procedure.

Synthesis of ***5c***. Hexamethylenediamine (5.89 g, 50.7 mmol) was dissolved in 35 mL of degassed water. **3b** (1.50 g, 0.88 mmol) was added at once and stirred at 37 °C under nitrogen for 19 h. The reaction was further carried out at 70 °C for 3 h then concentrated under reduced pressure. Cyclodextrins were precipitated with 11:1 acetone:methanol and collected by filtration. Ion-exchange chromatography yielded the pure product (855 mg, 54% yield). MALDI-TOF-MS $[M + H]^+ = 1493.7$.

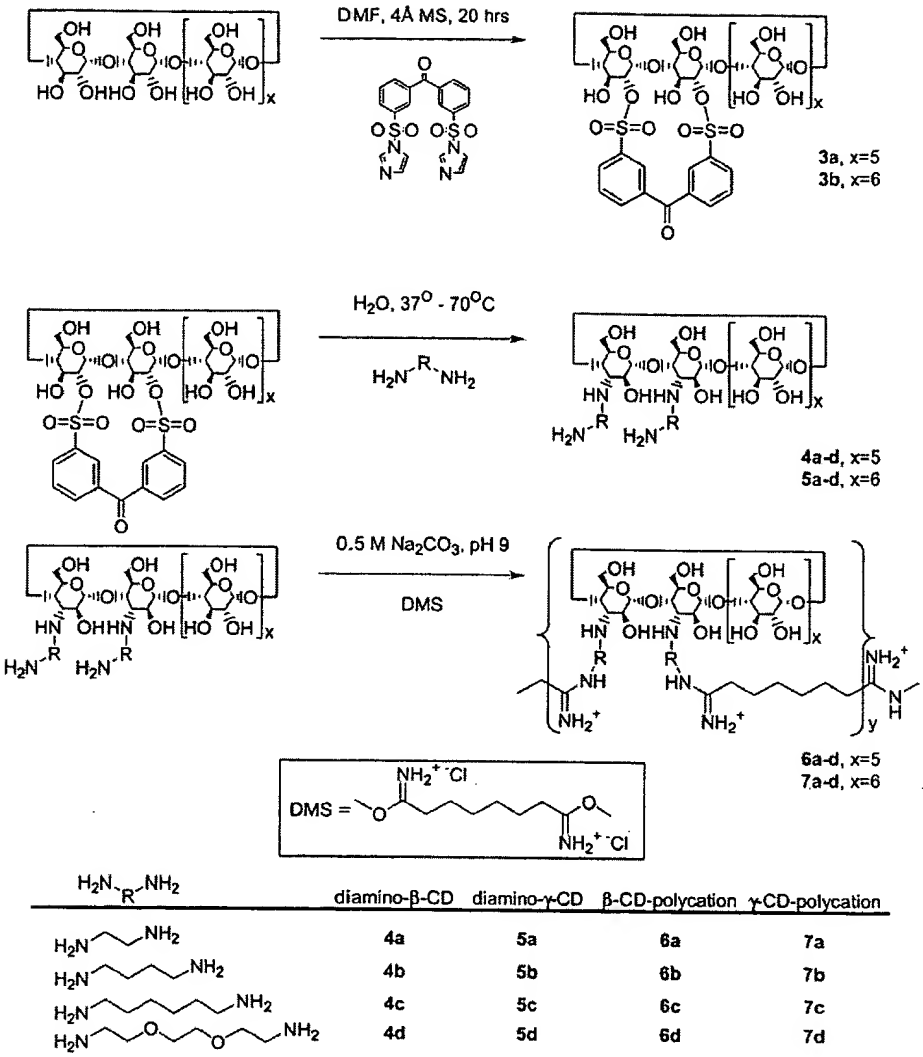
The polycations were prepared as exemplified by the following procedure.

Synthesis of ***7c***. ***5c*** (100 mg, 54.7 μmol) and DMS (15.5 mg, 56.7 μmol) were taken up in 108 μL of 0.5 M Na_2CO_3 and stirred for 13 h. Acidification with 1 N HCl to pH 2.0 followed by exhaustive dialysis yielded 58.4 mg of a white powder (56% yield).

Light Scattering and Molecular Weight Determination. The specific refractive index (RI) increment, dn/dc, of each polycation was determined by fitting a linear curve to plots of RI versus concentration (five data points per polycation). Polycations were then analyzed on a Hitachi D6000 HPLC system equipped with an ERC-7512 RI detector and a Precision Detectors PD2020/DLS light scattering detector using a PL aquagel-OH column (Polymer Laboratories, Amherst, MA). The eluent was 0.8 M ammonium acetate with 0.05% sodium azide, adjusted to pH 2.8 with phosphoric acid and flowing at 0.7 mL/min. RI values were measured on a Carl Zeiss refractometer (Max Erb Instrument Co., Burbank, CA) in the same eluent as used for HPLC analysis.

Plasmid DNA. Plasmid pGL3-CV (Promega; Madison, WI) was amplified with the DH5α strain of E. coli (Gibco

Scheme 2



BRL; Gaithersburg, MD) and purified using the Ultramobius 1000 kit (Novagen; Madison, WI). This plasmid encodes the firefly luciferase gene under control of the SV40 promoter.

Polyplex Formation and Characterization. Polyplexes were formulated by adding polycation solutions in dH_2O to an equal volume of plasmid DNA (pDNA) in dH_2O (0.05 mg/mL final pDNA concentration) and incubating for 30 min. Desired charge ratios were achieved by using appropriate concentrations of polycation solution. Each polycation was examined for its ability to bind pDNA through a gel electrophoresis assay using a 0.8% agarose gel (30 μg ethidium bromide/50 mL TAE buffer). Particle size and ζ-potential of polyplexes were analyzed using a ZetaPALS instrument (Brookhaven Instruments; Holtsville, NY).

Cell Culture and Transfections. BHK-21 cells were maintained at 37 °C in 5% CO_2 atmosphere in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 μg/mL amphotericin B (Gibco BRL). For transfections, cells were seeded at 50000 cells/well in 24-well plates. Trypan blue exclusion was used to verify cell viability above 95%. At 1 day, cells were exposed to 200 μL of serum-free medium containing 1 μg of pGL3-CV plasmid pre-assembled with CD-containing polycations at various charge ratios. After 4 h, polyplex solutions were removed from the cells and replaced with 1 mL of regular growth medium. For measurement of luciferase activity and toxicity, cells were lysed 2 days after transfection with 1X Cell Culture Lysis Reagent (Promega). The Luciferase Assay System (Promega) was used to measure luciferase activity of cell lysates on a Monolight 2010 luminometer (Becton Dickinson Biosciences; San Jose, CA). Total protein content of cell lysates was assessed with the DC Protein Assay (Bio-Rad; Hercules, CA) that is a derivative of the Lowry assay.

RESULTS

Polycation Synthesis and Characterization. β- and γ-CDs were selectively difunctionalized through a stapling reaction with benzophenone-3,3′-disulfonyl imidazole (Scheme 1). These intermediates react with various alkyl- and alkoxydiamines to yield $3^A,3^B$-bis(aminoalkylamino)-CDs with various spacing groups between the carbohydrate ring and the primary amine (Scheme 2). The difunctionalized amino-CD monomers were polymerized with DMS to give polycations with properties shown in Table 1. The choice of CD-comonomer influences the polymerization with DMS; polymerization yield increases with increasing distance between the cyclodextrin-ring and the primary amine on the CD-monomer. Similar yield trends were observed for otherwise identical β- and γ-CD polycation syntheses. CD-monomers with

Table 1. Effect of Cyclodextrin Comonomer Structure on Polymerization

polycation	polymerization yield (%)	d*n*/d*c* (mL/g)	M_w (kDa)	M_w/M_n	average degree of polymerization
6a	32	0.1029	10.0	1.1	6
6b	44	0.1406	8.1	1.3	5
6c	61	0.1515	13.9	1.7	8
6d	74	0.1322	13.0	1.4	7
7a	32	0.1085	9.3	1.1	5
7b	47	0.1386	9.6	1.4	5
7c	56	0.1237	14.7	1.6	8
7d	58	0.1279	13.3	1.3	7

Table 2. Particle Sizing and Zeta-Potential of Polycation/pDNA Complexes Formulated at Charge Ratio (±) of 5

polycation	particle diameter (nm)	zeta potential (mV)
6a	121.5 ± 1.3	12.5 ± 0.3
6b	96.4 ± 1.1	6.4 ± 1.1
6c	107.7 ± 0.9	16.7 ± 1.7
6d	88.2 ± 6.9	27.7 ± 1.0
7a	124.1 ± 1.6	23.3 ± 0.5
7b	118.6 ± 23.9	17.5 ± 3.0
7c	153.3 ± 1.7	9.6 ± 1.1
7d	102.9 ± 1.0	30.7 ± 1.4

fewer than four methylenes between the cyclodextrin and the primary amine yielded polycations with an average degree of polymerization (DOP) of 5 or 6, while those synthesized from monomers with over four spacer methylenes produced an average DOP of 7 or 8. An increase in polydispersity accompanied the increase in polycation length.

Polyplex Formation and Characterization. To demonstrate polycation interaction with pDNA, polyplexes were formulated and run on a 0.8% agarose gel at a range of charge ratios. Polycations **6a** and **7a** did not completely retard DNA below a charge ratio of 1.5, while **6b–d** and **7b–d** retarded DNA at charge ratios of 0.5 and above (Figure 2). The diameter of polycation/pDNA polyplexes varied between 100 and 150 nm, while the associated ζ-potentials were all found to be positive (Table 2).

In Vitro Transfection Efficiency. In vitro transfection efficiency to BHK-21 cells was assessed in triplicate at charge ratios (±) of 2, 4, 6, 8, 10, 15, and 20. Lysates of transfected cells were assessed for luciferase activity by measuring the relative light units (RLU) normalized by total protein content (Figure 3). Among the diaminoalkyl-CD analogues, **6a–c** and **7a–c**, increased spacer length produced greater transfection efficiency, with more pronounced enhancements between the **a** and **b** variants in each series. The diaminoalkoxy-CD analogues, **6d** and **7d**, demonstrated intermediate levels of luciferase expression, below that achieved with the **b** analogues. Generally speaking, the β-CD and γ-CD polycations with identical spacers produced similar luciferase gene expression.

In Vitro Cellular Toxicity. The total protein content of cell lysates was used as a measure of polyplex and/or polycation toxicity (Figures 4 and 5). The fractional cell survival of transfected cells was assessed by comparison to untransfected cells. Among the charge ratios investigated, polycations **6a–c** and **7a–c** demonstrated a marked decrease in cell viability with increased spacer length; **6d** and **7d** were essentially nontoxic at the concentrations employed. For the **b** and **c** analogues, cell viability decreased with increasing charge ratio and was worse for the β-CD polycations than for the γ-CD polycations. The toxicity of each polycation was independent of the presence of pDNA, as determined by comparison of polyplex-transfected cells with those exposed to an equal amount of polycation alone (Figure 5).

DISCUSSION

Previous studies of β-cyclodextrin-containing polycations (βCDPs) have demonstrated the importance of polycation structure to cellular toxicity and in vitro transfection efficiency. The effect of interamidine distance (*14*) has been elucidated, as has the importance of using a bulky cyclodextrin instead of a smaller carbohydrate such as trehalose (*15*). Here, we investigated the relevance of cyclodextrin ring size by studying otherwise identical series of β- and γ-cyclodextrin polycations. Within each series, the length and character of the spacer between the cyclodextrin ring and the amidine charge center were varied to understand the importance of these additional variables in our system. Such an approach allows the direct evaluation of the effect of cyclodextrin-type on in vitro transfection efficiency and cellular toxicity as well as providing further insights into the role of charge spacing along the polycationic backbone. For in vivo application of the polycations described in this report, modifications are required to impart salt and serum stability. Methodologies for modifying similar cyclodextrin-based polycations for in vivo use are available in our earlier publication (*20*).

The β- and γ-CD-based series of polycations follow remarkably similar trends in the DOP. DOP is found to increase with distance between the reactive primary amines of the CD-monomers and the cyclodextrins themselves. As the number of methylenes between the cyclodextrin and primary amine increases, an increase in DOP is observed with an accompanying increase in polydispersity. All polycations shown here have an average DOP between 5 and 8, corresponding to an average of 10–16 amidine charge centers per polycation chain. Assuming these differences in DOP do not significantly affect polycation performance, a direct correlation may be made between polycation structure and the observed performance.

Previous work demonstrated that the transfection efficiency and toxicity achieved with CD-containing polycations is affected by the presence of cyclodextrins and by the alkyl chain length between charge centers (*14*, *15*). Here, it is demonstrated that the transfection efficiency and toxicity of a related set of polycations is affected by the structure of the spacer separating the CD ring from the charge centers and, to a lesser degree, the type of CD used.

Diaminoalkyl-CD polycations **6a–c** and **7a–c** exhibit a marked increase in transfection efficiency as the spacer length increases, particularly with the increase from 2 to 4 methylene units. Dramatic differences between the **a** and **b** analogues are observed despite only a small change in polycation structure (a 2 Å increase in distance between the cyclodextrin and the amidine charge center). A smaller but significant increase in transfection efficiency is observed between the **b** and **c** analogues. Polycations **6a** and **7a** gave low levels of luciferase expression that gradually increased with increasing charge ratio. Optimum expression levels observed with these two polycations were of the same order as transfection efficiencies seen with polycations **6b**, **6c**, **7b**, and **7c** at the lowest charge ratios. Having reached relatively high transfection efficiencies at the lowest investigated charge ratios, polycations **6b**, **6c**, **7b**, and **7c** did not display the steady and marked increase with charge ratio

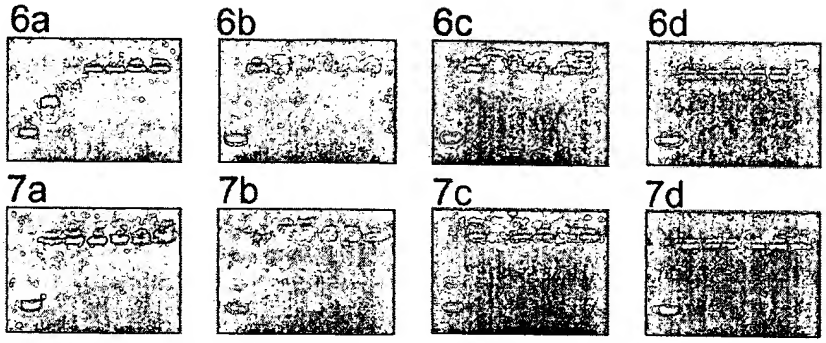


Figure 2. Agarose gel electrophoresis of polycation/pDNA complexes. For each polycation, complexes were formulated at charge ratios (±) of 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 and run in order of increasing charge ratio (left to right) on a 0.8% agarose gel.

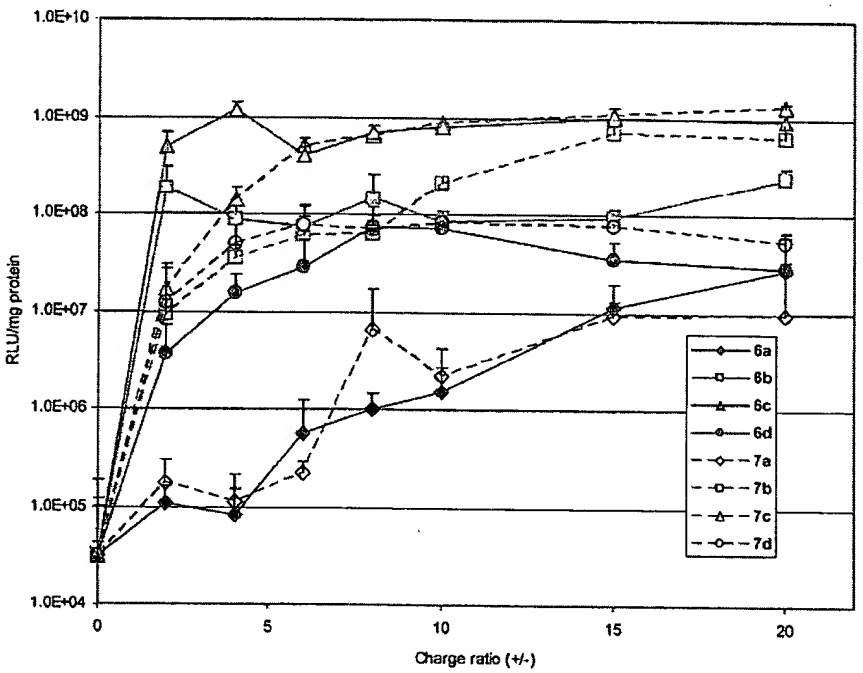


Figure 3. Relative light units (RLU)/mg protein as a function of charge ratio for cyclodextrin-polycation/pDNA complexes. Complexes were formulated at various charge ratios and exposed to BHK cells in serum-free medium for 4 h. 48 h after exposure, the cells were assayed for luciferase activity. Charge ratio of 0 indicates naked pDNA.

seen with **6a** and **7a**. Beyond a charge ratio of 6±, only **7b** demonstrated a significant increase in luciferase expression.

The least effective polycations, **6a** and **7a**, are also observed to require the highest charge ratio to completely retard pDNA in the electrophoresis assay. Previous work with CD-containing polycations has shown a correlation between relative binding efficiency and transfection efficiency (*15*). The reduced binding efficiency associated with decreased spacer length may result from the bulky cyclodextrins impeding the access of polycation amidines to pDNA phosphates.

The presence of CDs in the polycation backbone has been shown to produce a dramatic reduction in toxicity of β-CD-containing polycations (*13*–*16*). In part 1 of our study (15), $6^A,6^D$-dideoxy-$6^A,6^D$-diamino-β-CDs were studied, while $3^A,3^B$-dideoxy-$3^A,3^B$-diamino-β- and γ-CDs are investigated here. The transfection and toxicity assays employed in this series of papers do not indicate any advantages of functionalization of the CD at the C(3)-position over functionalization at the C(6)-position. In part 1 it was shown that longer spacer lengths between the CD and the charge center result in increased toxicity, that is in agreement with the result that polycations **6a**–**c** and **7a**–**c** demonstrate an increase in toxicity as the CD-amidine distance is increased. These results suggest that there is a toxicity-mitigating influence of the CD on the cationic center, regardless of the site of CD-derivatization. The CD may be affecting the interaction of the amidine charge centers with intracellular entities through its steric bulk and/or large sphere of hydration and thus lowering the toxicity of amidine-containing polycations. The bulkiness of the CD also hinders access of polycation amidines to pDNA phosphates. Since CD bulkiness and/or sphere of hydration correlate with the trends in both toxicity and transfection efficiency, the observation that decreases in toxicity are associated with decreases in transfection efficiency and effective pDNA binding strength are self-consistent.

The diaminoalkoxy-CD polycations **6d** and **7d** demonstrate an intermediate level of transfection efficiency and insignificant toxicity. Although this polycation pair provides the largest spacing between the CD and amidine residues among polycations in this study, the hydrophilic nature of the alkoxy spacer likely enlarges the effective hydration sphere around the cyclodextrin ring. In addition, the alkoxy spacer has more flexibility than alkyl spacers. These factors somehow mitigate the toxicity of

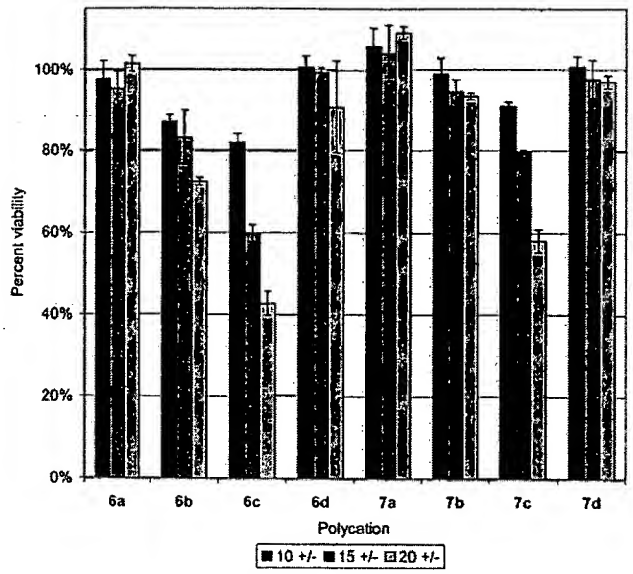


Figure 4. Cell viability to exposure of polycation/pDNA complexes at various charge ratios. Cells were assayed for viability 48 h after exposure to complexes; data were normalized with respect to untreated cells.

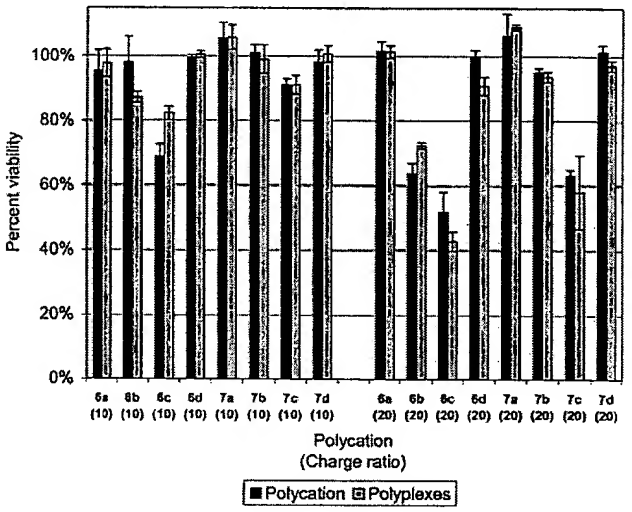


Figure 5. Comparison of the toxicity of polycation alone to polycation/pDNA complexes. Cells were exposed to polycation alone or to an equal amount of polycation complexed with pDNA; "charge ratio" for polycation alone merely represents amount of polycation. Total protein concentrations in cell lysates were used as measure of viability; data were normalized using values for untreated cells.

polycations **6d** and **7d**. The change in transfection efficiency as a function of charge ratio is also intermediate relative to the diaminoalkyl-CD polycations; RLU/mg protein readings with polycations **6d** and **7d** rose gradually up to a charge ratio of 6±, above which no increase is observed.

Each polycation produced measurable luciferase expression above background levels; the luciferase activities of untreated cells and cells treated with polycation alone are roughly 5×10^3 RLU/mg protein (data not shown) while the luciferase activity of cells treated with pDNA alone is roughly 5×10^4 RLU/mg protein. For comparison, BHK-21 cells were transfected with complexes of pDNA formulated with 25 kDa branched polyethylenimine or with βCDP6 (*14*). Polyethylenimine complexes at an N/P of 5 were found to give luciferase activity of 5×10^9 RLU/mg protein (data not shown). βCDP6 complexes produced 2×10^8 RLU/mg protein at a charge ratio of 10± (data not shown).

Here, analogous β- and γ-CD-containing polycations produced similar levels of gene expression, with the exception that polycations **6b** and **6c** outperform their γ-CD-containing analogues **7b** and **7c** at charge ratios of two and four; however, these differences do not persist as the charge ratio is increased. At higher charge ratios, toxicity differences between the β-CD-containing polycations **6b** and **6c** and the γ-CD-containing analogues become apparent, with the γ-CD-containing polycations being less toxic. It is again interesting to note the correlation between enhanced transfection efficiency and increased toxicity.

The peripheral diameter of γ-CD is about 17.5 Å while that of β-CD is about 15.4 Å (*19*), highlighting the importance of even small variations in the CD-containing polycation system to in vitro performance. Since the polycation backbone goes through adjacent sugar residues of the cyclodextrin ring in the case of the β- and γ-CD polycations discussed in this report, the linear backbone structure varies minimally between the two. However, the remainder of the cyclodextrin-ring, which can be considered pendant to the backbone, is certainly larger in the case of γ- over β-CD.

In part 1, Reineke and Davis showed that trehalose-based polyamidines are more toxic than those based on β-CD. Here, CD-containing polycations demonstrate an increase in toxicity with an increase in distance between the CD and the amidine charge center and with a decrease in the size of the CD-ring. Together, these results are consistent with the hypothesis that the size of the carbohydrate moiety and its associated sphere of hydration (overall increase in hydrophilicity) mitigate the toxicity of the amidine-based polycations.

We have described the synthesis and characterization of a family of cyclodextrin-containing polycations and demonstrated significant and clear effects of polycation structure on in vitro gene expression efficiency and cellular toxicity against BHK-21 cells. The structure of diaminated cyclodextrins was found to influence both the molecular weight and polydispersity of polycations resulting from reaction of these compounds with dimethyl suberimidate. Longer alkyl regions in the polycation backbone increased transfection efficiency and toxicity, while increasing hydrophilicity was toxicity-reducing. Further, γ-CD polycations were shown to be less toxic than otherwise identical β-CD polycations.

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Supporting Information Available: MALDI-TOF spectra of CD-monomers **4a–d** and **5a–d**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Cyclodextrin-Containing Polymers for Gene Delivery

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Key words: cyclodextrin, gene delivery, DNA, polymers

Abstract

Cyclodextrin-containing polymers are now being explored as vehicles for delivering nucleic acids into cells. The structures of the cyclodextrin-containing polycations affect the nucleic acid delivery efficiencies and their toxicities. Of interest is the fact that the cyclodextrin-containing polymers reveal lower toxicities than polymers that lack the cyclodextrins. The cyclodextrins endow the nucleic acid delivery vehicles with the ability to be modified by compounds that form inclusion complexes with the cyclodextrins, and these modifications can be performed without disruption of the polymer-nucleic acid interactions. Thus, cyclodextrin-containing polymers provide unique properties for gene delivery.

Introduction

The development of polyplexes (cationic polymer + nucleic acid) for gene delivery has grown at a rapid pace from initial work involving readily available polymers like poly-L-lysine (PLL) and polyethylenimine (PEI) to current studies that exploit polymers designed for this application. Cationic polymers are able to deliver DNA into cells by self-assembling with the anionic DNA via electrostatic interactions to subsequently form positively charged, small particles (sub-500 nm) that are taken up by cells.

While cationic polymers share a common mechanism of DNA delivery, their delivery efficiencies differ greatly from polymer to polymer. Additionally, significant variations in delivery efficiency and toxicity are observed by the use of various molecular weight fractions of the same polymer. Finally, little is known regarding the relationships between the molecular architecture of the polymer and the delivery properties of the polyplexes formed from that polymer.

Cyclodextrin-containing polymers have been known for quite some time. Examples of various classes of cyclodextrin-containing polymers are illustrated in Figure 1. In the mid-1990's, we began work on the synthesis of new cationic polymers for use as DNA delivery agents. We hypothesized that it may be possible to prepare low toxicity polycations from cyclodextrins [1] because numerous individual cyclodextrins (CD) were known to reveal low toxicity and to not elicit immune responses in animals. Additionally, cyclodextrins were exploited in drug formulation because of their ability to form inclusion complexes, and we planned on using this property in the assembly of fully formulated products that would be appropriate for systemic DNA delivery. In 1999, we reported on the synthesis of a new family of cyclodextrin-containing, cationic polymers (CDP)

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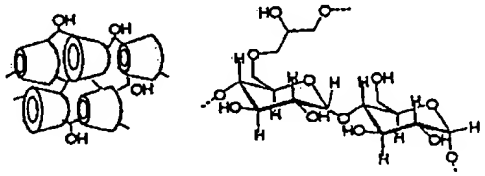
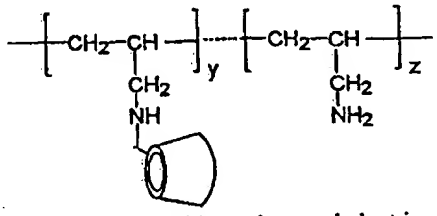


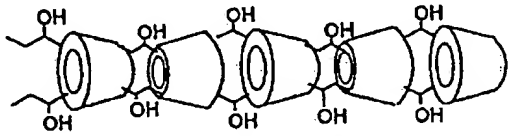
Cyclodextrin crosslinked with 1-chloro-2,3-epoxy propane

(b)



Polyallylamine with pendent cyclodextrins

(c)



Linear tube of cyclodextrins

Figure 1. Classes of cyclodextrin-containing polymers.

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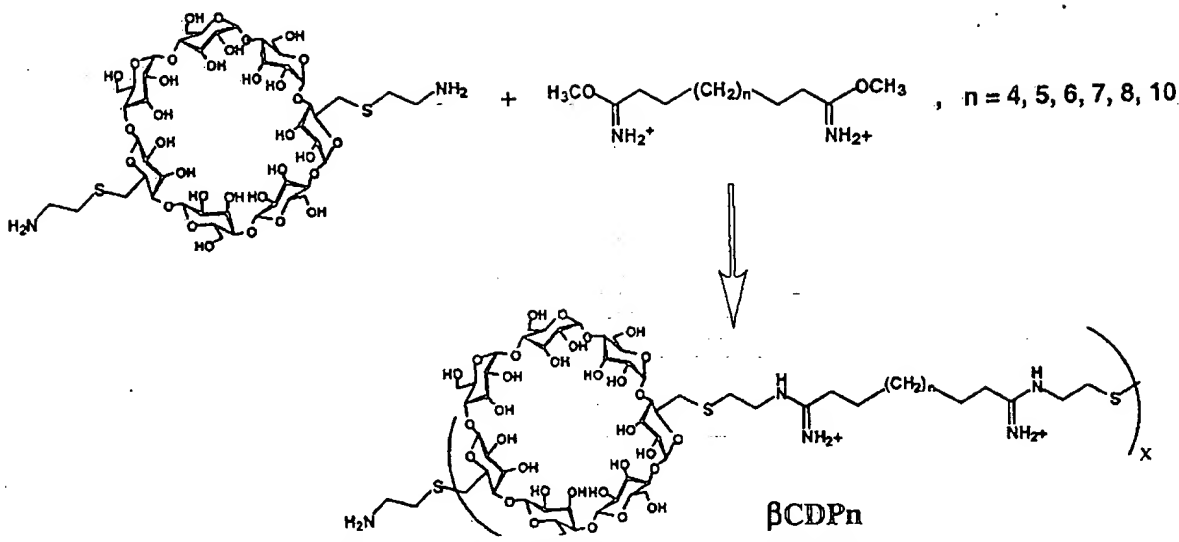


Figure 2. Polymerization scheme for β-cyclodextrin-containing polymers.

that were prepared by the condensation of difunctionalized CD monomers with other difunctionalized comonomers (see Figure 2) [1]. These linear polycations were able to provide effective DNA delivery to cultured cells with low toxicity [1, 2].

Numerous cyclodextrin-containing, cationic polymers currently exist. For example, within the class of cyclodextrin pendent polymers (see Table I), several are polycations, e.g. PEI, poly(allylamine), dendrimers. Although these materials have been known for some time, only recently has any of them been used to deliver genes to cultured cells. Arima *et al.* have delivered DNA to cells using α-, β- and γ-cyclodextrin-containing polyamidoamine dendrimers [3]. This work and ours with CDP are the only examples of cyclodextrin-containing polymers used for gene delivery.

Polyplex formulations optimized for *in vitro* delivery are typically not appropriate for *in vivo* use because successful systemic delivery requires different particle properties. After intravenous injection, cationic polyplexes interact with serum proteins and are quickly eliminated from the bloodstream by phagocytic cells. Additionally, the polyplexes rapidly aggregate at physiological ionic strength (150 mM salt concentration). Thus, cationic polyplexes require modification before they can be successfully applied for systemic gene delivery. A typical modification of polyplexes is to provide steric stabilization by PEGylating the particles (PEG: polyethylene glycol). Numerous methods are available for covalent attachment of PEG to create PEGylated particles..

The use of cyclodextrin-containing polycations for polyplex formation provides the means to create modified particles in an entirely new manner. Pun and Davis recently developed methodologies to modify the surface of polyplexes formed with cyclodextrin-containing polymers whether they be of the CDP-type [4, 5] or not [5]. This concept exploits the use of cyclodextrin/guest compound complexation to provide modified polyplexes appropriate for systemic application as gene delivery vehicles. As an example of this methodology, adamantane was conjugated to PEG and the resulting compound exposed to CDP-based polyplexes (see Figure 3) for self-assembly between the adamantane and the cyclodextrins. This methodology can provide CDP-based particles that are appropriate for systemic gene delivery [4].

In this paper, we discuss further issues: (i) associated with distributing charge centers along the CDP backbone, and (ii) modifying the surface of CDP-based polyplexes with adamantane-based compounds.

Materials and methods

All materials synthesis procedures and methods of characterization have been described previously, as have the cell transfection and toxicity protocols [1, 2, 4]. Further details on the surface modification compounds and their properties will be available shortly for cyclodextrin-containing polymers in general [5].

Results and discussion

Distribution of charge centers on CDP

Previously, Hwang *et al.* prepared a series of CDPs that varied the spacing between the charge centers by preparing polycations with spacer units containing 4–10 methylenes (see Figure 2) [2]. Table II shows the properties of these polymers and the polyplexes prepared from them. All the CDPs rapidly form polyplexes of approximately the same size. However, the gene delivery efficiencies as determined by luciferase gene expression assays and the cellular toxicities are strong functions of the spatial distribution of charge centers along the CDP backbone. The optimal gene delivery

Table 1. Examples of cyclodextrin pendent polymers

Type of polymer	Cyclodextrin	Preparation method
polyacrylic esters	α, β	polymerization of vinyl cyclodextrin derivatives
poly(allylamine)s	β	grafting of cyclodextrin to preformed polymer
acrylonitrile-methyl acrylate copolymer	β	grafting of cyclodextrin to preformed polymer
polymethacrylates	α, β, γ	polymerization of cyclodextrin methacrylate monomers
chitosan	β	grafting of cyclodextrin to preformed polymer
polyester	β	grafting of cyclodextrin to preformed polymer
polyethylenimine	β	grafting of cyclodextrin to preformed polymer
dendrimers	α, β, γ	grafting of cyclodextrin to preformed polymer

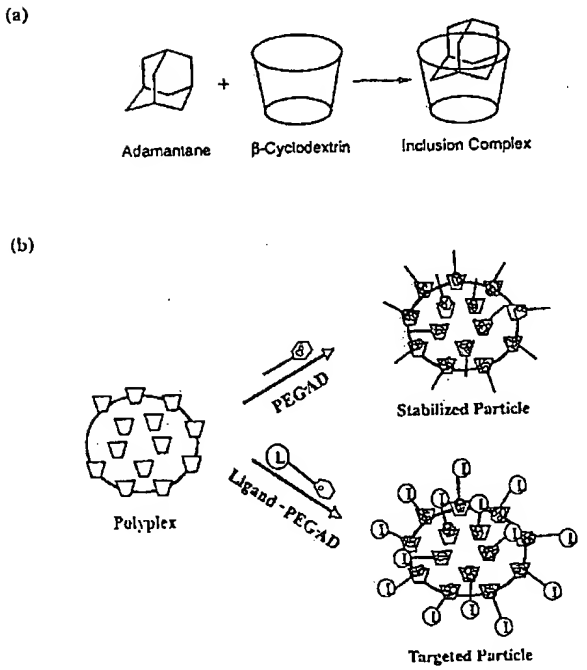


Figure 3. Schematic of (a) inclusion complex formation and (b) surface modification of cyclodextrin-containing polyplexes. From [4].

expression occurs with a CDP having 6 methylenes separating the charge centers (β-CDP6). As the distance between the charge centers is increased, the toxicity is diminished for spacings generated by 4–8 methylenes. The CDP with 10 methylenes spacing the charge centers becomes more toxic most likely because it reveals lower water solubility as compared to the other polycations [2]. Based on these results, two other CDPs have been prepared and they are schematically represented in Figure 4. β-CDP(NH) gives approximately the same gene delivery and toxicity properties as β-CDP6 while β-CD(NH)P6 shows significant cellular toxicity (see Figure 5). Thus, the spatial distribution of charge centers along the backbone of the CDP plays a significant role in the toxicity. We are currently attempting to understand the roles that charge distribution play in the cellular delivery of DNA by CDP-based polyplexes.

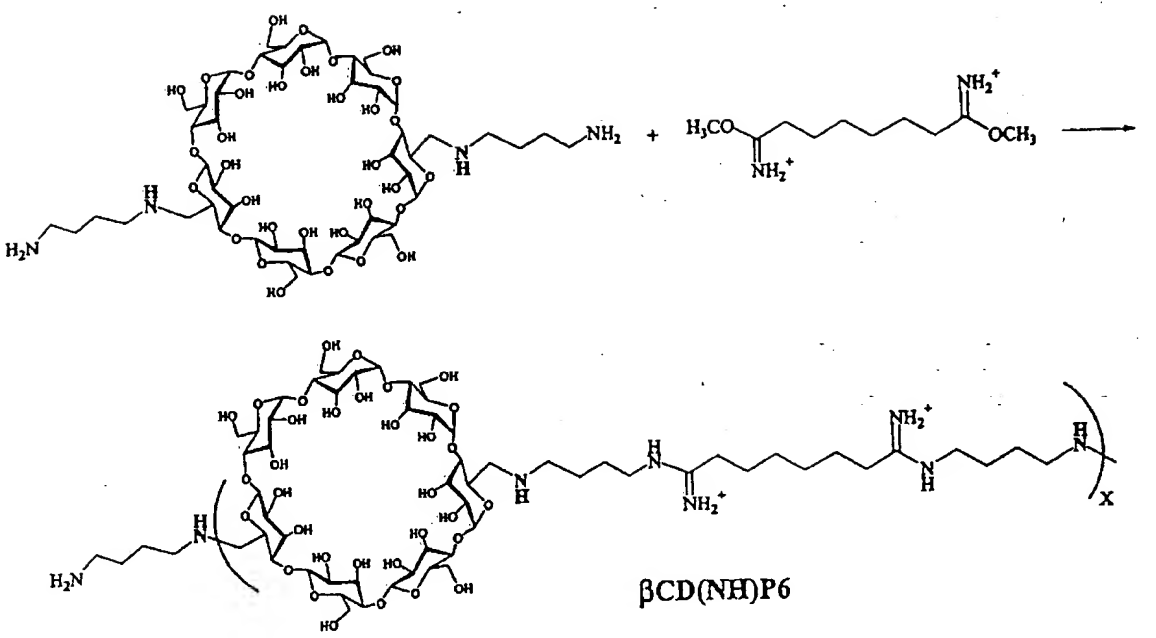
In addition to the charge distribution, the presence of the cyclodextrin has a significant effect on the toxicity of the linear polycations. For example, polyamidines (see Figure 6) prepared to mimic the charge distributions in CDPs reveal IC_{50}s of 0.005–0.034 mM at the conditions reported in Table II for the CDPs and a comparison of toxicities obtained from polyplexes prepared from these polycations is given in Figure 5. Thus, the cyclodextrin has a very large effect on the toxicity (or lack thereof) of the polycation.

Modification of polyplex surface

The concept of polyplex surface modification by entities that form inclusion complexes with the cyclodextrins of the cyclodextrin-containing polycations has recently been established [4, 5]. This method of polyplex modification does not involve the portions of the polycations that bind to the DNA so polyplex disruption is avoided. Although adamantane was initially used to form inclusion complexes with β-cyclodextrin-containing polycations [4], other combinations of guest species and cyclodextrin-types can be used [5]. The modifying agents contain PEG segments and can also contain anionic segments and targeting ligands at the opposite end of the adamantane [4, 5]. The targeting ligands used for binding to cell surface receptors can be small molecules, e.g. galactose, folate, and/or larger entities such as proteins [5].

The association between the adamantane-PEG (AD-PEG) molecules and the CDP-containing polyplexes was found to be quite strong and not what would be expected from the association of β-cyclodextrin and water-soluble adamantane analogues [6]. For example, CDP-containing polyplexes modified with AD-PEG_{5000} are stable in PBS (see Figure 7) and to dilutions in PBS to concentrations in the microgram/milliliter range. The high association may arise because of the very high local concentration of cyclodextrins on the polyplexes, additional interactions between the PEG and the cyclodextrins, e.g. hydrogen bonding, and/or to other factors. We are currently attempting to unravel the underlying mechanisms for the stability of the AD-PEG modified CDP-containing polyplexes.

It is clear that the inclusion complex formation method of surface modification can be applied to cyclodextrin-containing polyplexes in general and this has been accom-



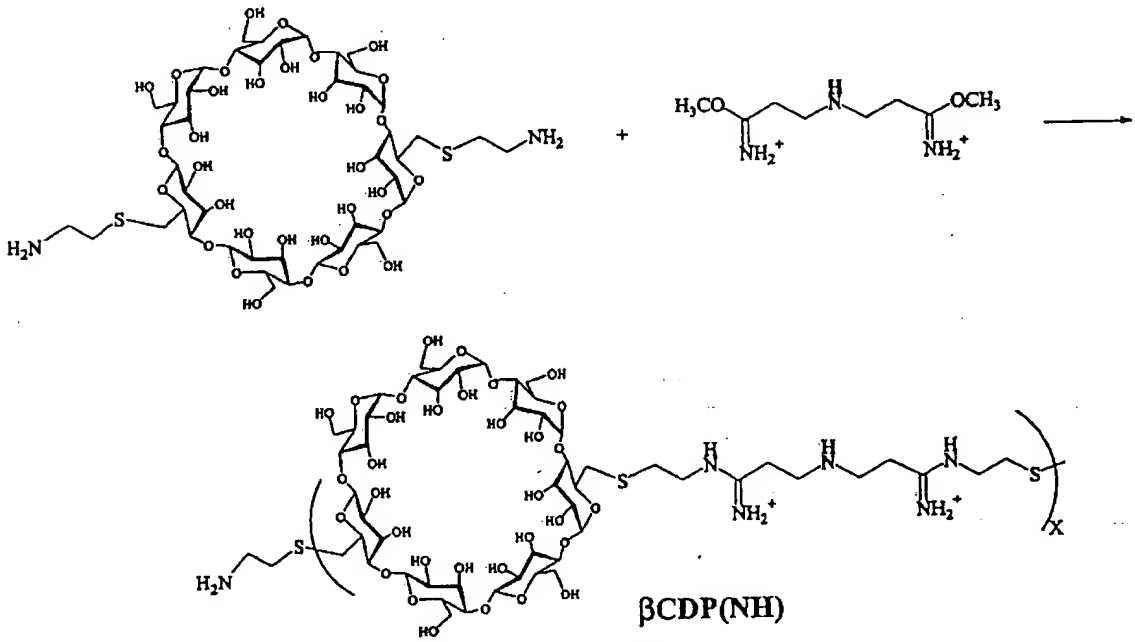


Figure 4. Further examples of linear, cyclodextrin-containing polymers.

Table 2. Effects of length between charge centers on CDPs [2]

No. of methylenes	M_w (kDa)	M_w/M_n	Polyplex size (nm)	Rel. gene Eff.[a]	IC_{50} (mM) [b]
4	6.1	1.13	148	0.22	0.4
5	5.8	1.12	140	0.05	0.4
6	5.8	1.12	128	1.00	1.1
7	6.9	1.14	130	0.50	1.8
8	7.6	1.16	125	0.64	2.2
10	10.1	1.21	142	0.10	0.3

[a] Relative transfection efficiency (see [2] for details).
[b] IC50 of polycation alone with BHK cells (see [2] for details).

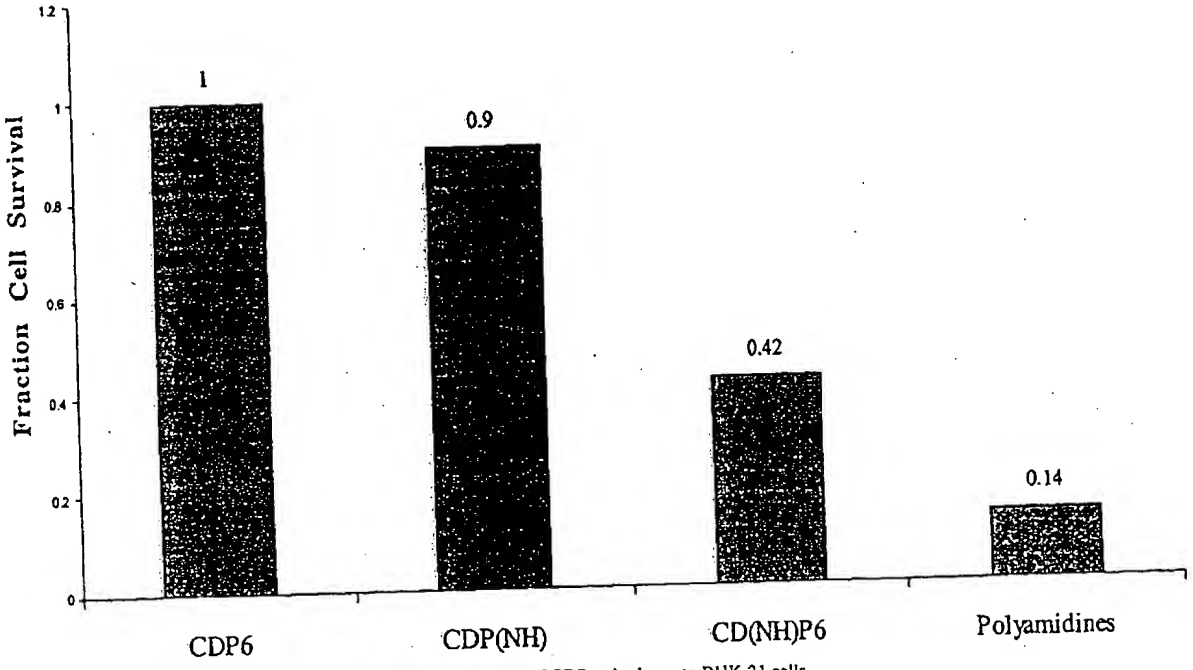
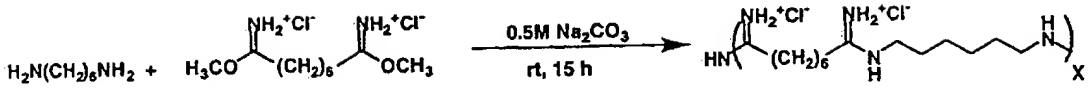


Figure 5. Toxicity of CDP polyplexes to BHK-21 cells.

C6–C6 Polycation:



C9–C6 Polycation:

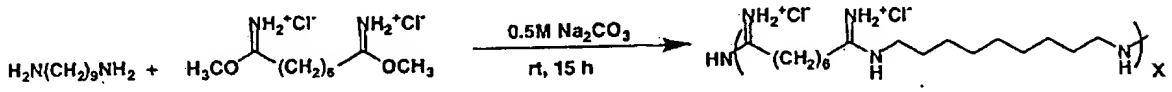


Figure 6. Polymerization scheme for non-cyclodextrin-containing polymers.

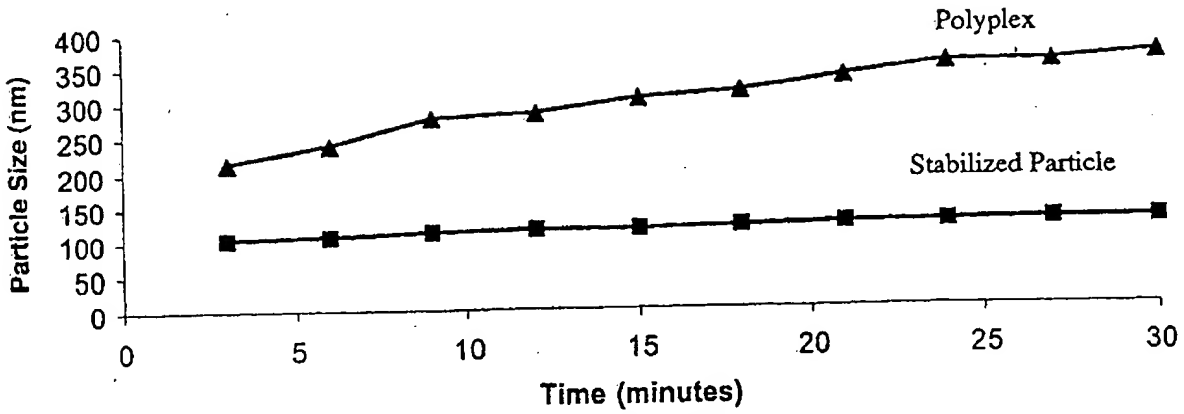


Figure 7. Particle size as a function of time in PBS.

plished for several types of cyclodextrin-containing polyplexes [5]. Complete characterization of these stabilized gene delivery vehicles is forthcoming.

Conclusions

Cyclodextrin-containing polymers are revealing new and exciting properties when used as gene delivery vehicles. The cyclodextrins endow the gene delivery vehicles with low toxicity and can serve as hosts that can form inclusion complexes with appropriate guest species to decorate the surfaces of polyplexes.

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The Effects of Structure on Gene Delivery with Linear β- and β-Cyclodextrin-Containing Polycations

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Key words: cyclodextrin diamines, polymerisation, toxicity, transfection efficiency

Abstract

Cyclodextrin (CD)-containing polycations are prepared by copolymerization of 3^A3^B-dideoxy-3^A,3^B-diamino-β- and γ-CDs with dimethyl suberimidate·2HCl to yield polyamidine products. Both alkyl- and alkoxy-diamines are used to vary the spacing between the CD and the amidine charge centers. It is found that the transfection efficiency and toxicity of such polycations is dramatically affected by the structure of the spacer separating the CD ring from the charge centers and less so by the type of CD used.

Introduction

Gene therapy holds tremendous promise for the treatment of ailments that are of genetic origin. Selected cationic lipids and polycations are currently under investigation as nonviral gene delivery vectors. The complexes produced through self-assembly of cationic lipids or polycations with DNA obviate the nucleic acid size limitations and immunological concerns of viral vectors, and large-scale synthesis of such complexes will likely be more cost effective than with viral vectors. Unfortunately, nonviral systems continue to fall short of viral-vector-mediated delivery efficiencies and most demonstrate unacceptable levels of toxicity for use as systemic delivery vectors in humans. In response to these shortcomings, chemical synthesis and rational design have increasingly been applied to the development of new nonviral gene delivery vectors. Recent reviews provide some information on observed structure-function relationships among nonviral vectors [1, 2].

A family of linear, β-cyclodextrin-containing polycations (β-CDPs) suitable for use as gene delivery vectors has been described [3]. The β-CDP materials were prepared by polymerization of 6^A-6^D-dideoxy-6^A,6^D-diamino-β-CDs with comonomers such as dimethyl suberimidate·2HCl (DMS) to give polyamidines. Initial structure-function studies with the β-CDPs showed the transfection efficiency and toxicity of these polycations to be a function of the intercharge spacing in the polycationic backbone [4]. Given this information, we expanded the scope of structure-function relationships for linear CD-containing polycations by synthesizing a larger and more diverse number of polycations. In this study, a new series of linear CD-containing polycations based on 3^A,3^B-dideoxy-3^A,3^B-diamino-β- and γ-CDs are explored. Additionally, alkyl- and alkoxy-diamines are used to vary the spacing between the CD and the amidine charge centers. This is also the first report of a linear, γ-CD-containing polycation.

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Experimental

Chemicals

β- and γ-cyclodextrins were purchased from Wacker Biochem Corp. (Adrian, MI) and dried in vacuo at 120 °C overnight before use. Chlorosulfonic acid (Alfa Aesar; Ward Hill, MA) was distilled before use. Dimethyl suberimidate·2HCl (DMS) was purchased from Pierce Endogen (Rockford, IL) and used without further purification. All other reagents were obtained from commercial suppliers and were used as received. Ion-exchange chromatography was run on a Toyopearl SP-650M (Toso-Haas; Montgomeryville, PA) column (NH_4^+ form) and products were eluted with aqueous ammonium bicarbonate up to 0.4 M. Thin-layer chromatography was performed on Silica Gel 60 F 254 plates (EM Separations Technology; Gibbstown, NJ) and compounds were eluted with 5:3:3:1 *n*-PrOH:AcOEt:H_2O:NH3(aq) and visualized by reaction with ninhydrin. Mass spectra were obtained on a Hewlett Packard 1100 Series LC/MSD operated in electrospray ionization mode. NMR spectra were recorded on a Bruker AMX500 spectrometer as dilute solutions of either D_2O or DMSO-d_6. Dialysis was carried out using a 3500 molecular weight cutoff regenerated cellulose dialysis cassette (Pierce Endogen). Plasmid pGL3-CV (Promega; Madison, WI) was amplified with the DH5α strain of E. coli (Gibco BRL; Gaithersburg, MD) and purified using the Ultramobius 1000 kit (Novagen; Madison, WI). This plasmid encodes the firefly luciferase gene under control of the SV40 promoter.

Synthesis

$2^A,2^B$-disulfonated-β-cyclodextrin, **3a** and *$2^A,2^B$-disulfonated-γ-cyclodextrin*, **3b** were synthesized according to literature methods [5, 6]. NMR and mass spectra data were in agreement with published values.

*Synthesis of $3^A,3^B$-di(aminoalkylamino)- and $3^A,3^B$-di(aminoalkoxyamino)-cyclodextrins (**4a–d** and **5a–d**)*
These syntheses were carried out as exemplified by the following procedure.

Synthesis of **5c**
Hexamethylenediamine (5.89 g, 50.7 mmol) was dissolved in 35 mL degassed water. **3b** (1.50 g, 0.88 mmol) was added at once and stirred at 37 °C under nitrogen for 19 hours. The reaction was further carried out at 70 °C for 3 hours then concentrated under reduced pressure. Cyclodextrins were precipitated with 11:1 acetone:methanol and collected by filtration. Ion-exchange chromatography yielded the pure product (855 mg, 54% yield). ESI-MS $[M + H]^+ = 1498$.

*Synthesis of polycations (**6a-d** and **7a-d**)*
These syntheses were carried out as exemplified by the following procedure.

Synthesis of **7c**
5c (100 mg, 54.7 μmol) and DMS (15.5 mg, 56.7 μmol) were taken up in 108 μL 0.5M Naa_2CO_3 and stirred for 13 hours. Acidification followed by exhaustive dialysis yielded 58.4 mg of a white powder (56% yield).

Light scattering and molecular weight determination

The specific refractive index (RI) increment, d*n*/d*c*, of each polycation was determined by fitting a linear curve to plots of RI versus concentration (five data points per polycation). Polycations were then analyzed on a Hitachi D6000 HPLC system equipped with a ERC-7512 RI detector and a Precision Detectors PD2020/DLS light scattering detector using a PL aquagel-OH column (Polymer Laboratories, Amherst, MA). The eluent was 0.8 M ammonium acetate with 0.05% sodium azide, adjusted to pH 2.8 with phosphoric acid and flowing at 0.7 mL/min. RI values were measured on a Carl Zeiss refractometer (Max Erb Instrument Co., Burbank, CA) in the same eluent as used for HPLC analysis.

Polyplex formation and characterization

Polyplexes were formulated by adding polycation solutions in dH_2O to an equal volume of DNA in dH_2O (0.05 mg/mL final DNA concentration). Desired charge ratios were achieved by using appropriate concentrations of polycation solution. Retardation of polyplexes was investigated by gel electrophoresis in a 0.8% agarose gel (30 μg ethidium bromide/50 mL TAE buffer). Particle size and ζ potential of polyplexes were analyzed using a ZetaPALS instrument (Brookhaven Instruments; Holtsville, NY).

Cell culture and transfections

BHK-21 cells were maintained at 37 °C in 5% CO2 atmosphere in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 μg/mL amphotericin B (Gibco BRL). For transfections, cells were seeded at 50,000 cells/well in 24-well plates. Trypan blue exclusion was used to verify cell viability above 95%. At one day, cells were transfected in serum-free medium with 1 μg pGL3-CV plasmid pre-assembled with CD-polycations at various charge ratios. After four hours, polyplex solutions were removed from the cells and replaced with 1 mL regular growth medium. For measurement of luciferase activity and toxicity, cells were lysed two days after transfection with 1X Cell Culture Lysis Reagent (Promega). The Luciferase Assay System (Promega) was used to measure luciferase activity of cell lysates on a Monolight 2010 luminometer (Becton Dickinson Biosciences; San Jose, CA). Total protein content of cell lysates was assessed with the DC Protein Assay (Bio-Rad; Hercules, CA), a derivative of the Lowry assay.

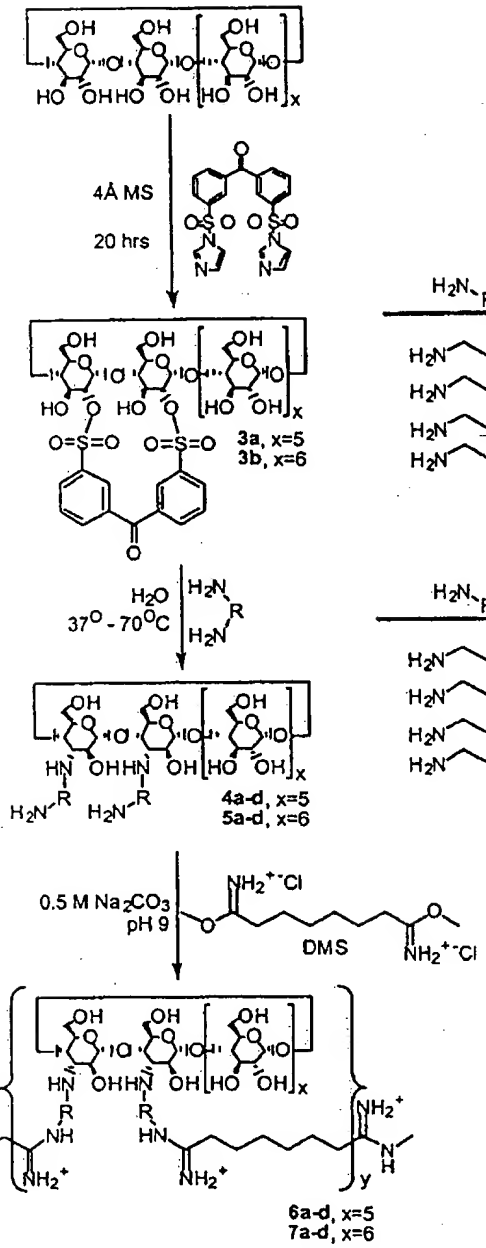
Results

Polycation synthesis and characterization

Cyclodextrin-polycations were synthesized using β- and γ-CDs functionalized with alkyl- and alkoxy-diamines (Scheme 1). The cyclodextrin-comonomer clearly influences the polymerization with DMS, as shown by the data given in Table 1. The polymerization yield increased with the distance between the cyclodextrin ring and the primary amine in the CD-monomer. Furthermore, the polymerization yield is quite similar for β- and γ-cyclodextrin-monomers with identical spacers. An increase in M_w is observed between cyclodextrin monomers having 4 or fewer methylene units between the cyclodextrin and the amidine and cyclodextrin monomers with longer spacers. This increase in polycation M_w is from an average degree of polymerization (DOP) of 5 or 6 to a DOP of 7 or 8. An increase in polydispersity accompanies the increase in M_w.

In vitro *transfection efficiency*

In vitro transfection efficiency was assessed at a charge ratio of 10 ±. Lysates of transfected cells were examined for luciferase activity by measure of relative light units (RLU) that were normalized by total protein content (Figure 1). An order of magnitude increase in transfection efficiency is observed as the length of the methylene spacer between the secondary amine and the charge center is increased from two to four, then again as the number of methylenes is increased from four to six. The diaminoalkoxy-CD analogues, **6d** and **7d**, demonstrated an intermediate level of transfection efficiency similar to **6b** and **7b**. No difference in transfection efficiency is observed between otherwise similar β- and γ-CD polycations. The luciferase activity of untreated cells, cells treated with polycation alone and cells treated with



H_2N–R–NH_2	diamino-β-CD	diamino-γ-CD
H_2N–(CH$_2$)$_2$–NH_2	4a	5a
H_2N–(CH$_2$)$_4$–NH_2	4b	5b
H_2N–(CH$_2$)$_6$–NH_2	4c	5c
H_2N–(CH$_2$)$_2$–O–(CH$_2$)$_2$–O–(CH$_2$)$_2$–NH_2	4d	5d

H_2N–R–NH_2	β-CD-polycation	γ-CD-polycation
H_2N–(CH$_2$)$_2$–NH_2	6a	7a
H_2N–(CH$_2$)$_4$–NH_2	6b	7b
H_2N–(CH$_2$)$_6$–NH_2	6c	7c
H_2N–(CH$_2$)$_2$–O–(CH$_2$)$_2$–O–(CH$_2$)$_2$–NH_2	6d	7d

Scheme 1. Synthesis of cyclodextrin-polycations.

Table 1. Effect of cyclodextrin comonomer on the polymerization

Polycation	Polymerization yield (%)	dn/dc (mL/g	M_w (kDa)	M_w/M_n	Average degree of polymerization
6a	32	0.1029	10.0	1.1	6
6b	44	0.1406	8.1	1.3	5
6c	61	0.1515	13.9	1.7	8
6d	74	0.1322	13.0	1.4	7
7a	32	0.1085	9.3	1.1	5
7b	47	0.1386	9.6	1.4	5
7c	56	0.1237	14.7	1.6	8
7d	58	0.1279	13.3	1.3	7

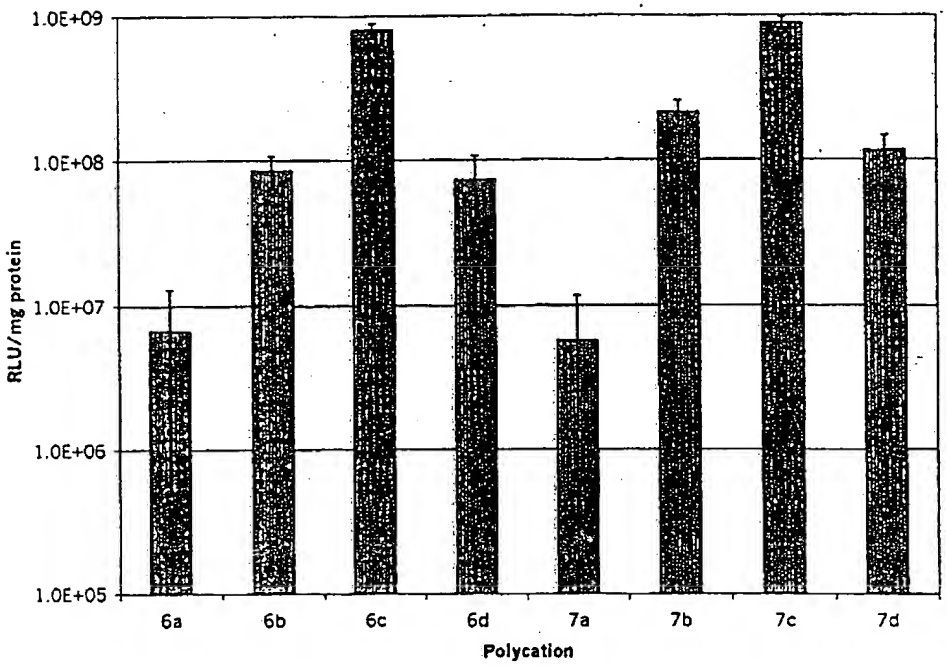


Figure 1. Transfection efficiency of polyplexes at a charge ratio of 10 ±.

DNA alone gave approximately 5×10^4 RLU/mg protein, which is significantly lower than the value obtained from cells treated with polyplexes.

In vitro *toxicity*

The total protein content of cell lysates was used as a measure of polycation toxicity. The fractional cell survival of transfected cells was assessed at a charge ratio of 10 ± by comparison to untransfected cells. Polycations **6c** and **7c** were considerably more toxic than the other polycations. Furthermore, polycation **6c** was more toxic than **7c**. Polyplex toxicity was found to be quite similar to toxicity of the same concentration of polycation without DNA as shown in Figure 2.

Discussion

The objective of this study is to elucidate the structure-function relationships for gene delivery with a family of linear β- and γ-cyclodextrin-containing polycations by investigating their ability to transfect cells *in vitro* with plasmid DNA. A series of cyclodextrin polycations was synthesized with various spacer-linkages between the cyclodextrin ring and the amidine charge centers. The type of cyclodextrin was also varied, such that all polycationic spacers have been prepared with both β- and γ-cyclodextrins. This approach allows direct evaluation of the effect of cyclodextrin-type on transfection efficiency and toxicity.

Polymerization yield increased with the distance between the cyclodextrin ring and the reactive primary amine of the cyclodextrin monomers with DMS. Similar yields were found for polymerization of the otherwise identical β- and γ-CD monomers. The steric bulkiness of the cyclodextrin could account for the reduced reactivity of the primary amines that are closer to the cyclodextrin cup. Thus, increasing the distance between the reactive center and the bulky cyclodextrin leads to a corresponding increase in the polymerization yield. Polycation molecular weights are found to increase by about 50% as the distance between the cyclodextrin and the reactive amine increases from 4 to 6 methylene units; an associated increase in polydispersity is also observed. The average degrees of polymerization of 5–8 correspond to an average of 10–16 cationic charges per polycation. If the differences in molecular weight are assumed to not significantly affect polycation performance, the variation in observed performance may then be attributed to differences in polycation structure.

Previous work demonstrated that the transfection efficiency and toxicity achieved with CD-containing polycations is affected by the presence of CD moieties and by the alkyl chain length between charge centers [4]. Here, it is demonstrated that the transfection efficiency and toxicity of such polycations is also affected by the structure of the spacer separating the CD ring from the charge centers and the type of CD used.

Transfection efficiencies of the polyplexes were found to increase as the length of the methylene spacer in the polycation increased, with the alkoxy derivatives **6d** and **7d** providing intermediate levels of transfection. Polycations **6c** and **7c**, which contain six-methylene spacers between the secondary amine and the amidine charge center, exhibit cell viability below that of the other polycations while providing the highest transfection efficiencies. Moreover, the γ-CD-

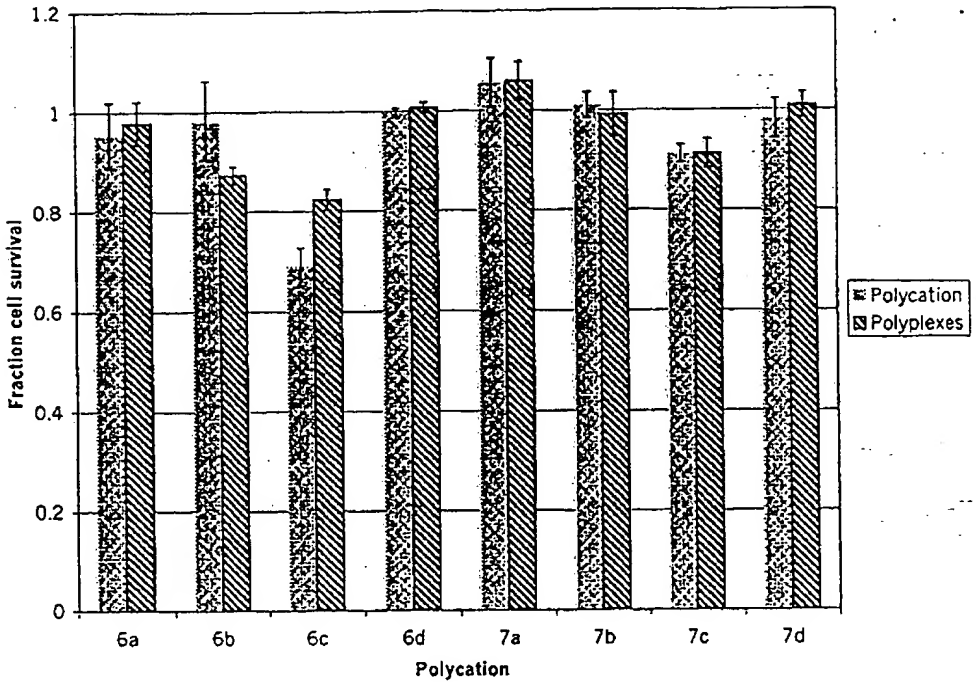


Figure 2. Toxicity of polyplexes (diagonally slashed bars) at a charge ratio of 10 ± and of polycation alone at the same polycation concentration in the polyplexes.

derivative, 7c, is less toxic than the β-CD-derivative, 6c. These results suggest that the toxicity-mediating influence of the CD may be a result of steric bulk, shielding intracellular entities from the otherwise toxic amidine charge centers. Toxicity of the polyplexes is largely independent of the presence of DNA and highly dependent on the concentration of polycation (Figure 2), suggesting that free polycation in solution should be minimized by formulating polyplexes at lower charge ratios.

From this initial study, it is clear that polycationic structure has a dramatic effect on performance. More detailed studies are underway and will be published later. Lower charge ratios will be investigated to attempt to show differences in transfection efficiency between the different polycations while likely reducing cell death due to lower concentration of polycation.

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